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ORIGINAL CONTRIBUTION

Calpains mediate isoproterenol-induced hypertrophy through modulation of GRK2

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Abstract

Inhibition of the Ca^{2+}-dependent proteases calpains attenuates post-infarction remodeling and heart failure. Recent data suggest that calpain activity is elevated in non-ischemic cardiomyopathies and that upregulation of the key cardiac G-protein-coupled receptor kinase 2 (GRK2) signaling hub promotes cardiac hypertrophy. However, the functional interactions between calpains and GRK2 in this context have not been explored. We hypothesized that calpain modulates GRK2 levels in myocardial hypertrophy of non-ischemic cause, and analyzed the mechanisms involved and the potential therapeutic benefit of inhibiting calpain activity in this situation. The oral calpain inhibitor SNJ-1945 was administered daily to male Sprague–Dawley rats or wild-type and hemizygous GRK2 mice treated with 5 mg/Kg/day isoproterenol intraperitoneally for 1 week. In isoproterenol-treated animals, calpains 1 and 2 were overexpressed in myocardium and correlated with increased calpain activity and ventricular hypertrophy. Oral co-administration of SNJ-1945 attenuated calpain activation and reduced heart hypertrophy as assessed using morphological and biochemical markers. Calpain activation induced by isoproterenol increased GRK2 protein levels, while genetic downregulation of GRK2 expression prevented isoproterenol-mediated hypertrophy independently of calpain inhibition. GRK2 upregulation was associated to calpain-dependent degradation of the GRK2 ubiquitin ligase MDM2 and to enhanced NF-κB-dependent GRK2 gene expression in correlation with calpain-mediated IκB proteolysis. These results demonstrate that calpain mediates isoproterenol-induced myocardial hypertrophy by modulating GRK2 protein content through mechanisms involving the control of GRK2 stability and expression. Sustained calpain inhibition attenuates isoproterenol-induced myocardial hypertrophy and could be an effective therapeutic strategy to limit ventricular remodeling of non-ischemic origin.

Keywords Calpain · Hypertrophy · GRK2 · Isoproterenol

Introduction

Cardiac remodeling occurring in response to a variety of pathologic insults is causally associated with the progression of heart failure (HF) [35]. The limited efficacy of the current pharmacological treatments to prevent this adverse outcome reveals an urgent need for novel targets and strategies aimed to limit ventricular remodeling secondary to different insults.

Calpains are a family of Ca^{2+}-dependent cystein proteases that participate in basic Ca^{2+}-mediated processes [9]. Among all the calpain isoforms identified, calpain-1 and calpain-2 are ubiquitously expressed and regulated by the intracellular Ca^{2+} concentration and by their endogenous inhibitor calpastatin. In pathological conditions associated with Ca^{2+} overload, as in myocardial reperfusion, a deregulated calpain activation takes

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place and contributes to myocardial injury through the proteolysis of different proteins [14].

In addition to its role in reperfusion injury, calpain-1 has been shown to participate in other situations resulting in ventricular remodeling and HF. Transgenic overexpression of calpain-1 in cardiomyocytes or restricted to mitochondria from cardiomyocytes, results in adverse remodeling and early mortality even in the absence of significant variations in intracellular Ca^{2+} [4, 6]. Calpain-1 overexpression has been observed in different experimental models of HF including chronic myocardial infarction and pressure overload induced by TAC or angiotensin II treatment [21, 36, 44], and in patients with congestive HF [50]. However, although most studies using transgenic animals with altered calpain/calpastatin system suggest that calpain overexpression may play an important role in the development of myocardial remodeling during chronic stress [21, 26, 36, 51], the evidence is not consistent [31, 45, 48].

Recently, we have demonstrated that calpain inhibition starting 24 h after transient coronary occlusion attenuates adverse post-infarction remodeling independently of its cardioprotective effects during the acute phase of reperfusion by modulating the hypertrophic and fibrotic response to a transient coronary occlusion [34]. However, only a limited number of studies have examined whether pharmacological inhibition of calpains prevents cardiac remodeling caused by non-ischemic pathologic stimulus, and the precise mechanism involved has yet to be determined [31, 49].

The myocardial protein content of the G-protein-coupled receptor kinase 2 (GRK2), a critical regulator of the cardiac G-protein coupled receptor (GPCR) signaling, is increased in patients with HF and in animal models subjected to chronic stress [12, 28, 47], and genetic ablation of GRK2 has been associated to improved cardiac function and reduced adverse post-infarction remodeling [37, 42]. Furthermore, different studies using transgenic models suggest the participation of GRK2 in the regulation of the signaling pathways linked to cardiac hypertrophy induced by TAC, angiotensin II and β-adrenergic stimulation [42, 52]. Altogether, these previous data raise the possibility of an interaction between calpains and GRK2 during the development of hypertrophy.

Therefore, in the present study we have determined whether βAR/calpain/GRK2 signaling networks interact to induce ventricular hypertrophy using a model of chronic stimulation of the β-adrenergic signaling with isoproterenol and tested the potential therapeutic efficacy of inhibiting calpains with a sustained oral administration of a calpain inhibitor.

Methods

Experimental protocol

The experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication Eighth Edition, 2011), and were approved by the Research Commission on Ethics of the Hospital Vall d'Hebron.

Male Sprague–Dawley rats weighing 250–300 g were used in these experiments. Chronic β-adrenergic activation was achieved by administration of isoproterenol at a dose of 5 mg/kg given intraperitoneally daily for 7 days (ISO group). In a group of rats receiving isoproterenol, the calpain inhibitor SNJ-1945 (SENJU Pharmaceutical Co., Ltd.,120 mg/kg + 0.5% carboximethyl cellulose) was orally co-administered (ISO+SNJ group). In the control group, rats received an intraperitoneal injection of the saline vehicle and oral administration of 0.5% carboximethyl cellulose. After 7 days of treatment, animals were sacrificed by intraperitoneal dose of sodium pentobarbital (100 mg/kg) and previous subcutaneous injection of buprenorphine (0.1 mg/kg). Hearts were excised, mounted in a Langendorff apparatus, perfused with Krebs buffer for blood removal and stored at − 80 °C or fixed in paraformaldehyde.

Male wild type (WT) and GRK2 hemizygous ($GRK2^{+/-}$) mice generated as previously described [25], were maintained on a C57BL/6 genetic background. $GRK2^{+/-}$ and their corresponding WT littermates (10–12 weeks) were treated with isoproterenol with and without the co-administration of SNJ-1945 as described above.

Echocardiographic analysis

Transthoracic echocardiography was performed in rats at baseline and at the end of isoproterenol treatment using a Vivid Q portable ultrasound system equipped with a i12L-RS 13 MHz transducer (GE Healthcare) as described earlier [34]. The left ventricular end-systolic (LVESd) and diastolic (LVEDs) internal diameters, interventricular septum thickness at end diastole (IVS) and left ventricular posterior wall thickness at end diastole (LVPW) were measured in M-mode recordings. Ejection fraction (EF) was calculated according to standard formulas. Images were analyzed off-line by an investigator blinded to the groups. For each parameter, measurements were performed from three to six different cardiac cycles, and the values were averaged.

Histological measurements

Hearts were fixed in buffered 4% paraformaldehyde and embedded in paraffin for histological evaluation. Mean cardiomyocyte cross-sectional area was measured in transverse sections stained with hematoxylin and eosin. At least 50 random cells from each heart ($n = 5$–6 per group) were measured at 400 × magnification.

In vitro determination of calpain activity

Frozen myocardium was homogenized in ice-cold Tris-buffered saline containing 1% Triton X-100 and centrifuged for 15 min at 15,000×*g*. Calpain activity was measured in the supernatant using the peptide substrate Suc-LLVY-AMC (Calbiochem). The release of the fluorescent product AMC was quantified with a multilabel reader (excitation, 360 nm; emission, 460 nm). The calpain inhibitor SNJ-1945 (10 µmol/L) was used to determine the specificity of the assay.

RT-PCR

Total RNA was extracted from heart samples using TRIsureTM reagent (BIOLINE, UK) following the manufacturer's protocol. Quantitative RT-PCR was performed using Taqman universal PCR master mix (Applied Biosystems, USA). The amplification program consisted of 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min and was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). Primers used for ANP, BNP, calpain-1, calpain-2, calpastatin, Myh6, Myh7 and GADPH were purchased from Thermofisher (Rn00561661_m1, Rn04219558_g1, Rn00569689_m1, Rn00567422_m1, Rn00583952_m1, Rn00691721_g1, Rn01488777_g1 and Rn01775763_g1, respectively). For GRK2 self-designed probes were purchased from Sigma labeled with Syber Green (Table 1) and POWER SYBR Green PCR Master Mix (Applied Biosystems, USA) was used with an amplification program consisting in 94 °C for 3 min, 40 cycles of 94 °C for 45 s, 55 °C for 30 s, 72 °C for 1 min and 72 °C for 10 min.

Table 1 Primers used for GRK2 RT-PCR analysis

BLDIS1 GRK2	Forward	5′-GGATCGAGGAGAAGTGACCTT-3′
	Reverse	5′-TGGCCTCTTCCAGATGGTT-3′
BIS2 GRK2	Forward	5′-CATGCACAATCGCTTTGTAGTC-3′
	Reverse	5′-AGGCCCAGGTCTGAGATTC-3′
DIS1 GAPDH	Forward	5′-TGCACCACCAACTGCTTAGC-3′
	Reverse	5′-TGGTCATGAGCCCTTCCAC-3′

Western blot analysis

Myocardial samples were homogenized and proteins separated by SDS-PAGE for Western blot analysis as previously described [11]. Primary antibodies used were raised against calpain-1 (Calbiochem), calpain-2 (Abcam), calpastatin (Abcam), CUL4A (Santa Cruz), DDB1 (Santa Cruz), Gβ (Santa Cruz), IκB (Cell Signaling), MDM2 (Santa Cruz), ERK1/2 (Santa Cruz), α-fodrin (Enzo), α-HMC (Abcam), α-SMA (Sigma-Aldrich), β-MHC (Abcam), GAPDH (Genetex) and Histone H3 (Abcam). GRK2 protein was detected with various specific rabbit polyclonal antibodies targeting different regions of the GRK2 (Fig. 4): AbFP2, raised against amino acids 436–689 [29], Ab 792, raised against residues 533-544 [7], and Ab C-15 (sc-562, Santa Cruz) that recognizes residues 675-689. GRK2 protein was normalized to total ERK1/2 levels. Protein bands were detected by chemiluminiscence (SuperSignal West Dura Extended Duration Substrate, Pierce) and quantified using a charge-coupled device system (Image Reader LAS-3000, Fujifilm) and image analysis software (Image Gauge, Fujifilm).

Analysis of MDM2 ubiquitination

Polyubiquitination levels of Mdm2 were analyzed using agarose-TUBEs (LifeSensors, Inc) following the manufacturer's protocol. Pulled-down samples (50 mg of rat hearts) were analyzed by immunoblotting using the anti-MDM2 antibody (N20 polyclonal rabbit 1:500, Santa Cruz) and then a specific polyubiquitin FK2 antibody (monoclonal 1:500, Enzo).

Analysis of cardiac tissue-polyubiquitinated GRK2

Frozen myocardium (80 mg) was homogenized and resolved in 7.5% non-reducing SDS-PAGE as previously described [40]. Ubiquitin conjugates of GRK2 were detected by immunoprecipitation of GRK2 protein with a monoclonal antibody (clone C5/1.1, Upstate) [40], followed by immunoblotting with an anti-ubiquitin antibody (Sigma) that recognizes all forms of mono- and polyubiquitin chains conjugated to proteins. After stripping, blots were incubated with a specific anti-K48-linkage polyubiquitin antibody (Cell Signaling) to detect polyUb chains targeting GRK2 for degradation by the proteasome. Blots were reprobed with a rabbit-specific anti-GRK2 antibody (sc-562, Santa Cruz) to detect total immunoprecipitated protein. Ubiquitination data were normalized by immunoprecipitated GRK2. Levels of GRK2 in whole-tissue extracts of each experimental group were confirmed by Western blot using specific antibodies.

Modulation of GRK2 transcriptional activity

We used human peripheral blood lymphocytes to investigate the modulation of GRK2 transcriptional activity. Peripheral blood T lymphocytes (PBTs) were obtained from buffy coats of healthy donors. The purity of the PBT population was always higher than 95% CD3+ cells. When indicated, cells were stimulated with the PKC activator Phorbol 12-myristate 13-acetate (15 ng/mL, Sigma-Aldrich) and the Ca^{2+} ionophore A23187 (1 µmol/L, Sigma-Aldrich). Viability of the cells at the concentrations used was confirmed by the trypan blue dye exclusion test. Transcriptional activity was measured using a luciferase GRK2 promoter construct in the pGL3 basic reporter plasmid containing a 0.9 kb-long proximal region of the human GRK2 gene (pGRK2-0.9) [38]. The pGRK2-0.9 reporter construct was transfected in PBTs in the absence or presence of an IκBα pCDNA3 expression plasmid [2], a generous gift of Dr J. Alcamí (CBMSO, Madrid). For transfection, cells were resuspended with 10 µg of plasmid DNA. In co-transfection experiments, 0.5 µg/mL of the correspondent empty or expression plasmid was included. After electroporation, cells were cultured for 16 h and then treated with different stimuli as indicated for 6 h, harvested by centrifugation and lysed. Luciferase activity was determined using a luciferase assay kit (Promega) with a luminometer Monolight 2010 (Analytical Luminescence Laboratory). Transfection experiments were performed with PBTs from at least three different donors.

Statistical analysis

Data analysis was performed using SPSS for Windows. Mean values between groups were compared by one-way ANOVA. Least significant difference test was applied as post hoc test when significant differences were observed. Unpaired *t* test was used in Fig. 7c. Linear regression analysis was used to analyze the correlation between mRNA levels of calpain-1 and markers of hypertrophy in Fig. 2d. All results are expressed as mean ± SEM.

Results

Chronic isoproterenol induces myocardial calpain upregulation in rats

Calpain-1 and calpain-2 mRNA content was elevated in rats treated with isoproterenol (Fig. 1a) and correlated with increased protein content (70% and 46% vs. control group; Fig. 1b), with no significant effect of SNJ-1945 treatment. The mRNA levels of calpastatin, the endogenous calpain inhibitor and also a calpain substrate were similar in all groups, while its protein levels were reduced in rats receiving isoproterenol but not in ISO + SNJ-treated animals (Fig. 1a, b). In agreement with such calpain and calpastatin protein expression patterns, the maximal calpain activity measured in heart homogenates and the in situ calpain activity, evaluated by the presence of proteolytic fragments (145/150 kDa) of the calpain substrate α-fodrin, were elevated in isoproterenol-treated rats (Fig. 1c). Importantly, oral administration of SNJ-1945 attenuated total calpain activity measured in vitro as well as α-fodrin and calpastatin degradation (Fig. 1b–d), confirming the efficacy of the drug and route of administration in preventing calpain activation.

Oral, chronic administration of SNJ-1945 attenuates isoproterenol-induced hypertrophy

Acute mortality in the isoproterenol group reached 10%. After 1 week of isoproterenol administration, echocardiography revealed cardiac hypertrophy as reflected by decreased ventricular internal dimensions and marked increase in LV posterior wall and septum thickness, heart weight/body weight ratio (Table 2 and Fig. 2a), and cardiomyocyte cross-sectional area (Fig. 2b). Daily oral co-administration of SNJ-1945 induced a significant attenuation in all these parameters. Providing further evidence of an isoproterenol-induced hypertrophic phenotype that is counteracted upon calpain inhibition, the increased β-MHC/α-MHC ratio, ANP and BNP mRNA levels observed after isoproterenol administration were attenuated with sustained calpain inhibition (Fig. 2c). Conversely, these hypertrophic markers showed a linear correlation with calpain-1 mRNA levels upon chronic isoproterenol treatment ($r^2 = 0.88$, $p < 0.001$ for β-MHC/α-MHC ratio; $r^2 = 0.89$, $p < 0.001$ for ANP, Fig. 2d).

Isoproterenol-induced upregulation of GRK2 protein content is prevented by calpain inhibition

Because GRK2 has been proposed to play a relevant role in cardiac hypertrophy, we next analyzed its protein levels in our different experimental groups using three antibodies targeting different regions of the protein (Fig. 3a). After 7 days of isoproterenol administration, the protein expression patterns detected by all the different antibodies showed a significant increase in myocardial GRK2 protein content that was prevented by sustained calpain inhibition (Fig. 3b). These data suggested that GRK2 upregulation might be a relevant hypertrophy-promoting event downstream isoproterenol-stimulated calpain overactivation. Therefore, we evaluated the effects of isoproterenol administration and calpain inhibition in previously characterized global hemizygous GRK2 mice ($GRK2^{+/-}$) [25], and in their respective control littermates (WT). In WT mice, chronic isoproterenol increased the heart weight/tibia length ratio as well as the β-HMC/α-HMC ratio and ANP mRNA levels thus confirming

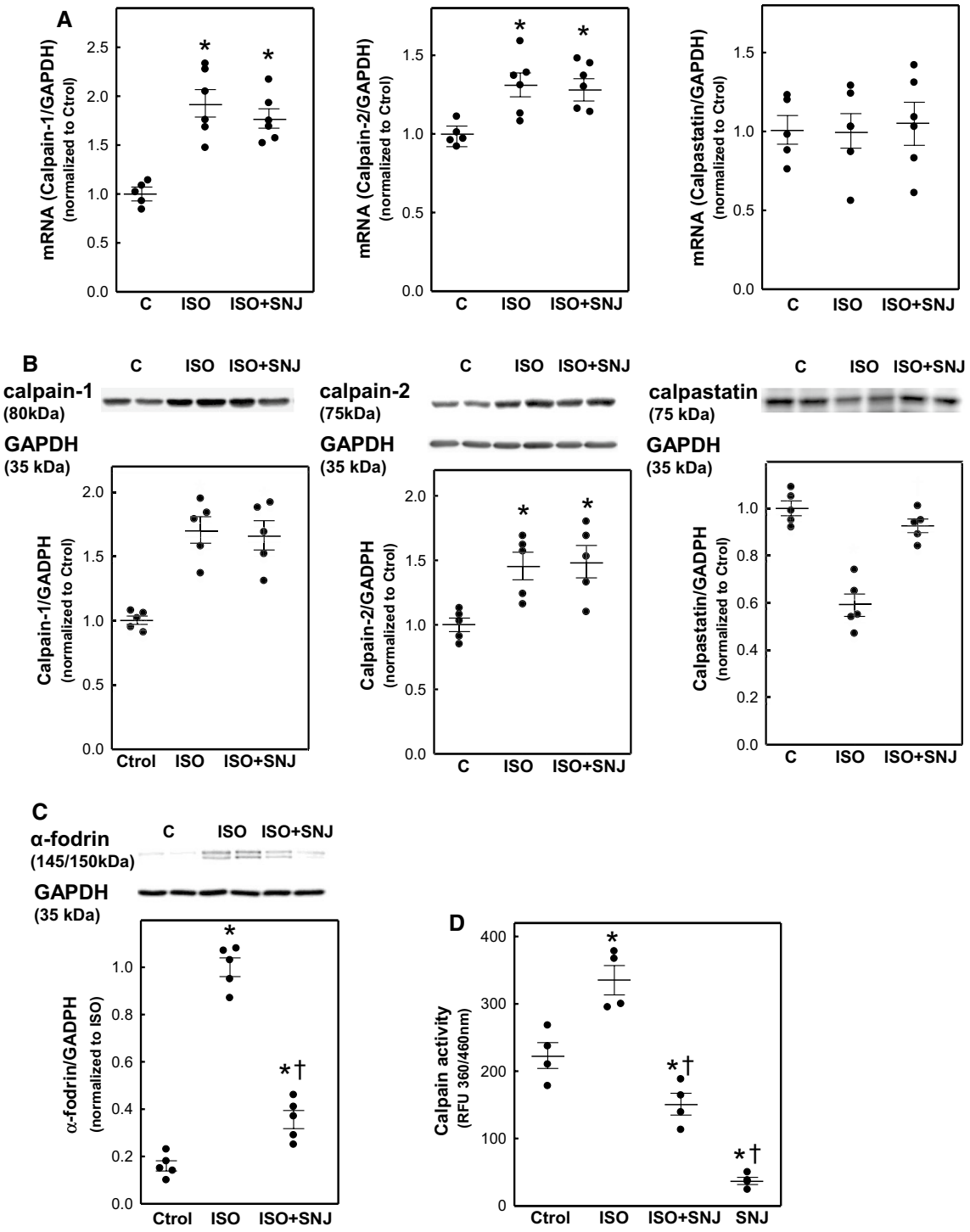

Fig. 1 Chronic isoproterenol treatment induces calpain overexpression. **a** Cardiac mRNA levels of calpain-1, calpain-2 and calpastatin, **b** representative western blot and quantitative densitometric analysis of calpain-1, calpain-2, calpastatin protein levels and **c** 145/150-kDa α-fodrin fragments in the indicated control (C), isoproterenol (ISO) and isoproterenol + SNJ-1943 (ISO+SNJ)-treated experimental groups. **d** Maximal calpain activity measured in vitro using homogenates obtained from hearts from the different experimental groups. SNJ: 10 µmol/L SNJ-1943 was added to homogenates from control hearts. Results are relative to GADPH, expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\dagger}p < 0.05$ vs. ISO group. $n = 4$–6 per group

Table 2 Weight and echocardiographic data

	Control ($n=6$)	ISO ($n=9$)	ISO+SNJ ($n=8$)
BW (g)	332 ± 12.5	336.2 ± 13.8	330.1 ± 8.8
HW (g)	1.07 ± 0.02	1.38 ± 0.02*	1.18 ± 0.04*†
HW/BW × 100	0.33 ± 0.02	0.41 ± 0.01*	0.36 ± 0.01†
HR (bpm)	342 ± 17	362 ± 12	348 ± 9
LVESD (mm)	3.9 ± 0.12	2.77 ± 0.14*	3.47 ± 0.24†
LVEDD (mm)	7.29 ± 0.16	6.01 ± 0.17*	6.71 ± 0.18*†
LVPWT (mm)	1.81 ± 0.12	2.78 ± 0.12*	2.30 ± 0.12*†
SWT (mm)	1.60 ± 0.09	2.66 ± 0.05*	2.17 ± 0.15*†
EF (%)	82.33 ± 1.92	78.46 ± 2.32	85.47 ± 2.25

BW body weight, *HW* heart weight, *HR* heart rate, *LVESD* left ventricular end-systolic diameter, *LVEDD* left ventricular end-diastolic diameter, *LVPWT* left ventricular posterior wall thickness, *SWT* septum wall thickness, *EF* ejection fraction

Mean ± SEM, *$p<0.05$ vs. control group. †$p<0.05$ vs. ISO group, analyzed by one-way ANOVA with LSD test

myocardial hypertrophy (Fig. 4a–c). Calpain-1 was overexpressed upon isoproterenol administration (Fig. 4d) and its inhibition with daily oral co-administration of SNJ-1945 significantly attenuated hypertrophy (Fig. 4a–c), reinforcing our results obtained in rats. Notably, however, the genetic downregulation of GRK2 in GRK2$^{+/-}$ animals markedly attenuated myocardial hypertrophy induced by isoproterenol and no additional effect was observed with the sustained inhibition of calpains (Fig. 4a–c). Interestingly, calpain-1 remained upregulated in GRK2$^{+/-}$ mice treated with isoproterenol, supporting the notion that GRK2 effects on hypertrophy are downstream calpain activation and that a calpain/GRK2 axis plays a relevant role in isoproterenol-promoted hypertrophy.

Calpain activation upregulates GRK2 by both decreasing its degradation machinery and by favoring its mRNA expression

GRK2 is a short-lived protein that undergoes polyubiquitination and degradation by the proteasome [32, 33]. To determine whether calpain upregulates GRK2 by reducing its degradation, we analyzed the ability of purified calpain to degrade different E3 ubiquitin ligases present in myocardial extracts that have been proposed to target GRK2 for proteolysis [32, 52], and whose potential downregulation by calpain would foster GRK2 stability. Our results showed a significant reduction in the protein levels of the MDM2 ubiquitin ligase in myocardial samples exposed to activated calpains in in vitro conditions that was prevented by the inhibition of calpains (Fig. 5a). This pattern was similar to that observed for the cleavage of α-fodrin, used as a control of calpain activation. In contrast, calpain activation did not induce any significant change in the protein content of G protein subunit β (G_β), CUL4A or DDB1 (Fig. 5a). Importantly, calpain-dependent reduction in the levels of MDM2 was also observed in myocardial samples obtained from isoproterenol-treated rats but not in those from rats receiving the co-administration of SNJ-1945 (Fig. 5b). Because MDM2 polyubiquitination by the ligase itself or by other factors can lead to increased MDM2 turnover [18], we next explored whether calpain activity might modulate MDM2 protein stability in an ubiquitin-dependent manner. Endogenously polyubiquitinated proteins were specifically pulled down from cardiac extracts using the tandem ubiquitin-binding entities (TUBEs) molecular tool, and probed with antibodies specific for MDM2. Of note, calpain inhibition prevented the increased MDM2 polyubiquitination observed in isoproterenol-treated animals (Fig. 6a). Overall, our data suggest that upon isoproterenol treatment, enhanced calpain activity might lead to decreased MDM2 levels via direct partial cleavage of MDM2 or by promoting the proteolytic clearance of unidentified MDM2 regulatory factors, resulting in increased polyubiquitination patterns of MDM2 and its degradation, while the presence of the calpain inhibitor prevents these events.

Consistent with the notion that MDM2 is key to promote ubiquitin-dependent degradation of GRK2, the polyubiquitination status of GRK2 was notably reduced along with decreased Mdm2 levels in isoproterenol-treated hearts compared to control samples, which display a strong signal of smeared bands in the 120–200 kDa range corresponding to polyubiquitinated GRK2 species, and this effect was attenuated upon administration of the calpain inhibitor (Fig. 6b). Of note, isoproterenol treatment also promoted a decay in the levels of GRK2 with ubiquitin chains conjugated via lysine-K48 linkages (Fig. 6c), which is the chief ubiquitination signal recognized by the proteasome and also the main ubiquitination pattern of myocardial GRK2 depending on MDM2 activity [17]. By contrast, calpain inhibition increased the extent of both high-order K48-ubiquitin GRK2 (above 150 kDa) and of GRK2 species with two–three ubiquitin moieties (circa 100 kDa) (Fig. 6c). Although the global levels of K48-polyubiquitinated GRK2 are similar in control conditions and in isoproterenol plus SNJ-1945-treated hearts (Fig. 6c), the patterns of K48-linked ubiquitin chains in the latter condition display species with low number of ubiquitin molecules (similar to those detected with antibodies directed against bulk polyubiquitin-conjugated chains shown in Fig. 6b) that might be less efficiently degraded by the proteasome. Altogether, these results suggest that the upregulation of GRK2 induced by isoproterenol treatment involves a defective proteasomal degradation of this protein by the MDM2-ubiquitin axis.

To explore the possibility that calpain activation might also increase GRK2 protein levels by modulating its transcription, myocardial GRK2 mRNA levels were measured

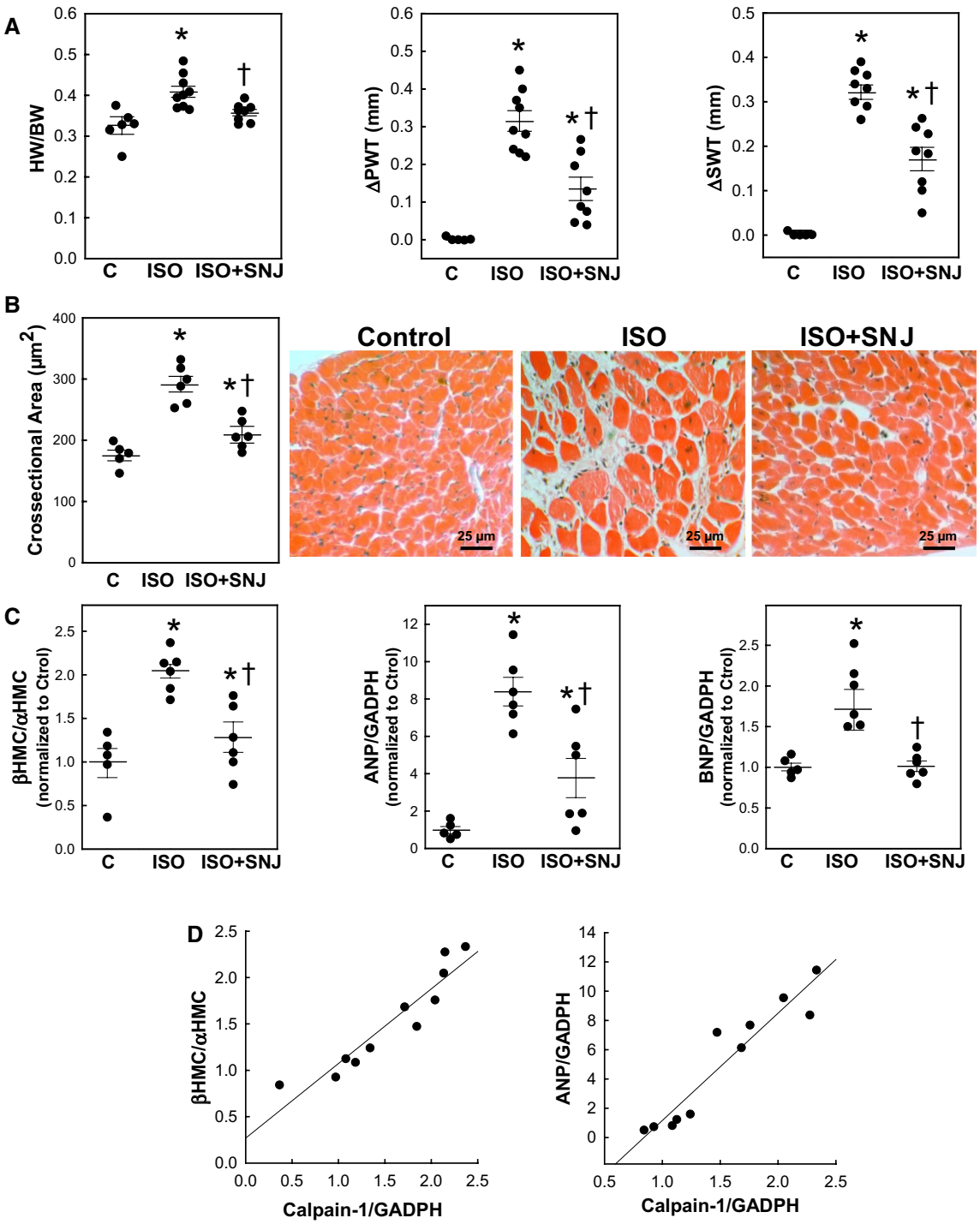

Fig. 2 Oral SNJ-1945 treatment attenuates isoproterenol-induced cardiac hypertrophy. **a** Hearts from the different experimental groups were analyzed for: heart weight/body weight ratio (HW/BW) and increase in septum (ΔSWT) and posterior wall (ΔPWT) thickness ($n=5$–8 rats per group); **b** ventricular cross-sections from these hearts were stained with hematoxylin and eosin followed by quantification of cardiomyocyte cross-sectional area (50 random cells/heart, $n=5$–6 hearts per group). Representative images shown. **c** mRNA β-MHC/mRNA α-MHC ratio and mRNA levels of ANP and BNP were normalized with respect to the control group ($n=5$–6 hearts per group). Results are presented as mean ± SEM. $^{*}p<0.05$ vs. control group. $^{\dagger}p<0.05$ vs. ISO group. **d** Linear correlation between mRNA levels of calpain-1 and markers of hypertrophy (mRNA β-MHC/mRNA α-MHC ratio and ANP mRNA), $n=5$–6 hearts per group

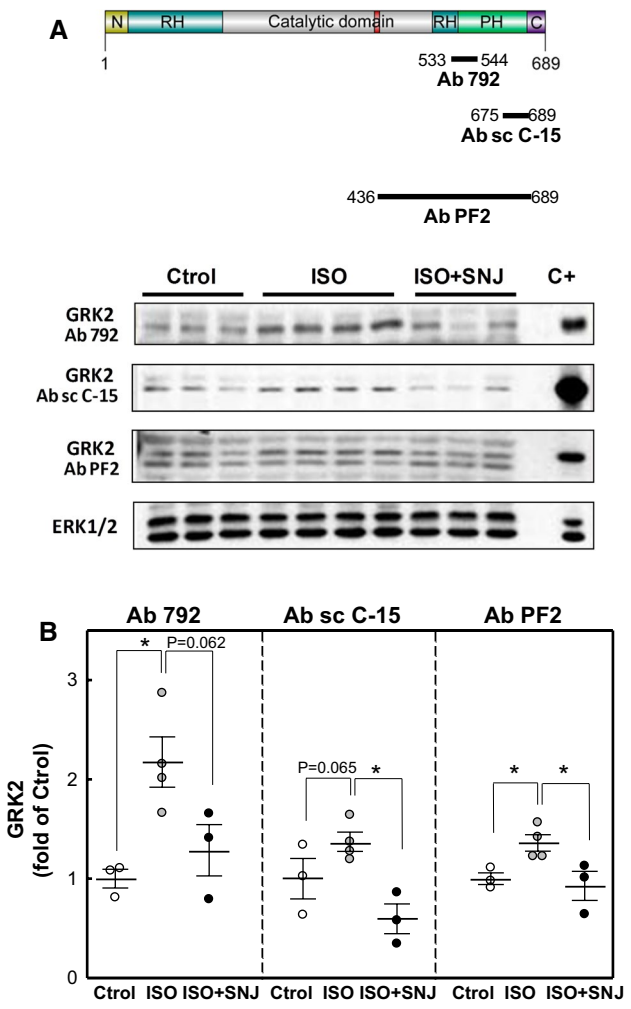

Fig. 3 Isoproterenol-induced upregulation of GRK2 protein content is prevented by calpain inhibition. **a** Myocardial extracts from control, ISO, and ISO+SNJ-treated rats were partially enriched in GRK2 protein for immunoblotting and analyzed using different antibodies raised against the indicated GRK2 regions. **b** Results were normalized to total ERK1/2 and expressed as fold change compared to control ($n = 3$–4 per group). A representative blot is shown. Data are presented as mean ± SEM. *$p < 0.05$ vs. ISO group

using two different primers. We found that isoproterenol treatment significantly enhances GRK2 gene expression while the co-administration of the calpain inhibitor prevents this effect (Fig. 7a). In search of potential mechanisms underlying such isoproterenol-induced and calpain-mediated increase in GRK2 mRNA levels, we noted that the 5′-flanking region of the proximal GRK2 human gene promoter contains consensus sequences for putative response elements of the NFκB transcription factors (Fig. 7b). Interestingly, activation of this nuclear factor has been related to isoproterenol-induced cardiac hypertrophy [5], and suggested to regulate the transcription of the GRK5 isoform in myocytes [16], whereas IκB, which restricts the activation of the NFκB pathway, is a known calpain substrate [34]. Thus, we explored the modulation of the transcriptional activity of the human GRK2 promoter by the NFκB pathway by expressing a luciferase reporter construct in human peripheral blood lymphocytes as a suitable heterologous experimental system. GRK2 expression in human peripheral lymphocytes mirrors changes in myocardial GRK2 levels during HF [13], and it has been proposed to have prognostic value in patients with HF [39]. Cell treatment with a combination of stimuli known to trigger NFκB stimulation [27], triggered a marked induction of GRK2 human promoter activity, which was significantly inhibited by co-transfection of the inhibitory construct IκBα (Fig. 7c), consistent with a role for the NFκB cascade in GRK2 transcription. In line with these data, we found that myocardial IκB is proteolysed when exposed to activated calpains in vitro (Fig. 7d) and that a significant calpain-dependent degradation of IκB is observed in myocardial extracts of rats upon chronic isoproterenol treatment (Fig. 7e), strongly suggesting a functional link between NFκB activity and GRK2 expression in this context.

Discussion

The present study proposes calpain-dependent upregulation of GRK2 protein levels, through both reduced degradation and increased synthesis, as a new mechanism of myocardial hypertrophy induced by chronic isoproterenol administration, and suggests that pharmacological calpain inhibition may prevent cardiac remodeling of non-ischemic cause.

Increasing evidence demonstrates that calpains are involved in cardiac remodeling occurring in response to chronic stress [22, 31, 49]. Previous studies using animal models with reduced calpain activity, by deletion of calpains 1 and 2 [26], or overexpression of calpastatin [51], have reported attenuated ventricular remodeling and dysfunction in experimental models with permanent coronary occlusion, while enhancing calpain activity by deletion of calpastatin had the opposite effect [20]. In contrast to post-infarction remodeling, the evidence supporting the contribution of calpains to cardiac remodeling in other common pathologic models of HF is more controversial. Overexpression and activation of calpain-1 have been described in myocardial samples from patients with congestive HF [49, 50] and in mice and rats subjected to aortic constriction [36, 49], receiving chronic administration of angiotensin II [21] or isoproterenol [3, 49]. However, although calpain inhibition by forced overexpression of calpastatin reduced angiotensin-induced myocardial and perivascular inflammation and fibrosis [21], it has been associated with the development of dilated cardiomyopathy [6]. Furthermore, while the use of a non-specific calpain inhibitor suggested the contribution of calpains to isoproterenol-induced hypertrophy [1],

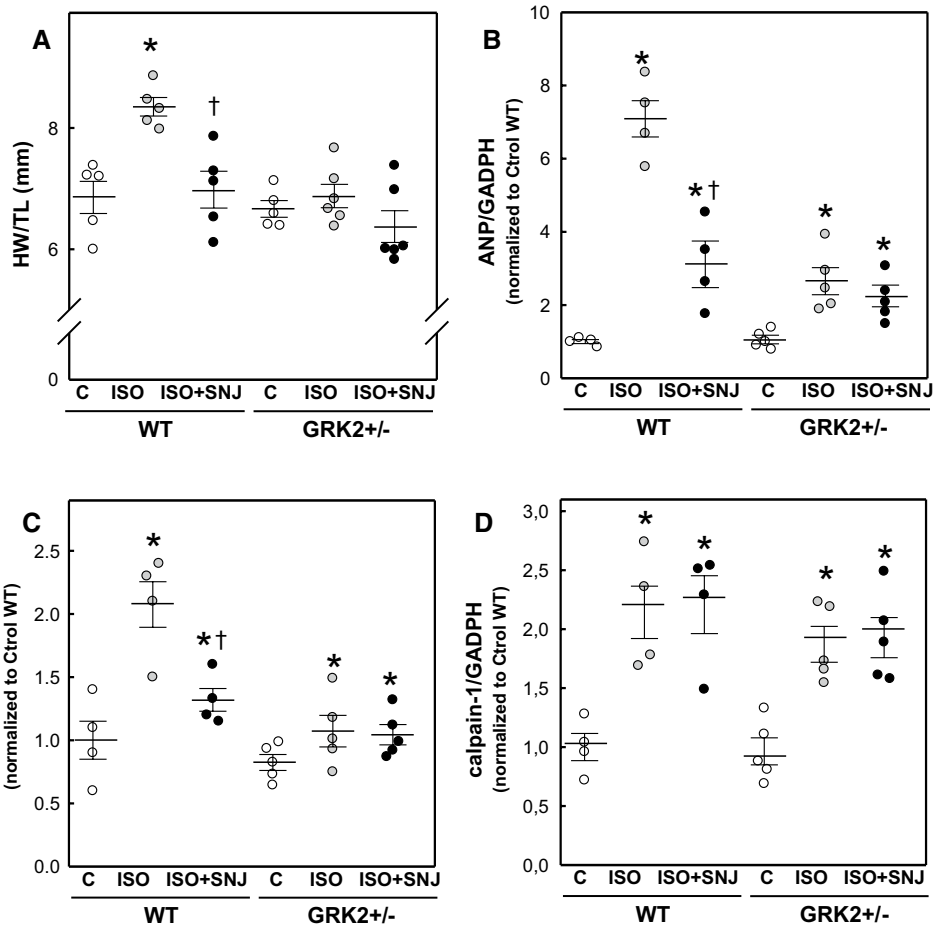

Fig. 4 Calpain contributes to isoproterenol-induced hypertrophy by upregulating GRK2 protein content. **a** heart weight/tibia length ratio (HW/TL), **b** mRNA β-MHC/mRNA α-MHC ratio, **c** mRNA levels of ANP and **d** mRNA levels of calpain-1, in wild type (WT) and GRK2 hemizygous (GRK2$^{+/-}$) mice receiving vehicle (C), or isoproterenol (ISO) with and without the co-administration of SNJ-1945 (ISO+SNJ group). mRNA levels are normalized with respect to WT control (C) group ($n = 4$–5 hearts per group). Results are presented as mean ± SEM. *$p < 0.05$ vs. control group. †$p < 0.05$ vs. ISO group

cardiac-specific knockout of the subunit Capn4, shared by calpain-1 and calpain-2, increased ventricular remodeling and dysfunction in mice subjected to either chronic isoproterenol treatment or TAC [45].

Despite the evidence on the pathophysiological importance of calpains in ventricular hypertrophy, there is a lack of studies testing the potential of pharmacological calpain inhibition as a therapeutic strategy for the treatment of ventricular remodeling [31, 49]. In our study we analyzed the effect of oral administration of SNJ-1945, a third-generation calpain inhibitor designed to improve its water solubility and bioavailability [30], in the cardiac hypertrophy induced by chronic administration of the β-agonist isoproterenol. Sustained adrenergic activity in the myocardium has been implicated in the development of pathological hypertrophy and progression of HF in animal models and humans [23]. In our study, and in agreement with previous reports, isoproterenol at 5 mg/Kg/day for 1 week, induced cardiac hypertrophy without producing significant cardiac dysfunction [41]. In this model we observed calpain activation associated to an increased expression of calpain-1 and calpain-2, with a close correlation between the expression of hypertrophic markers and calpain-1.

Importantly, daily oral administration of SNJ-1945 effectively inhibited myocardial calpain activity and markedly attenuated cardiomyocyte hypertrophy in both rats and mice. Although it has been suggested that some calpain inhibitors also inhibit MMP-2, which shares protein targets with calpain, previous results from our group exclude the possibility that the effects on hypertrophy obtained with SNJ-1945 were consequence of MMP-2 inhibition [34].

Different studies demonstrate that GRK2 is overexpressed in models subjected to chronic stress [12, 28, 47], and participates in the development of myocardial remodeling. In this regard, genetic inhibition of GRK2 activity prevented hypertrophy in response to TAC or β-adrenergic stimulation [42, 52]. In line with these studies, our results show that isoproterenol increases GRK2 protein levels. More importantly, sustained calpain inhibition prevented upregulation of GRK2, while the hemizygous deletion of GRK2 markedly attenuated myocardial hypertrophy induced by isoproterenol without altering calpain expression. Overall, these results strongly suggest that GRK2 upregulation is a relevant event in cardiac hypertrophy downstream isoproterenol-induced calpain overactivation.

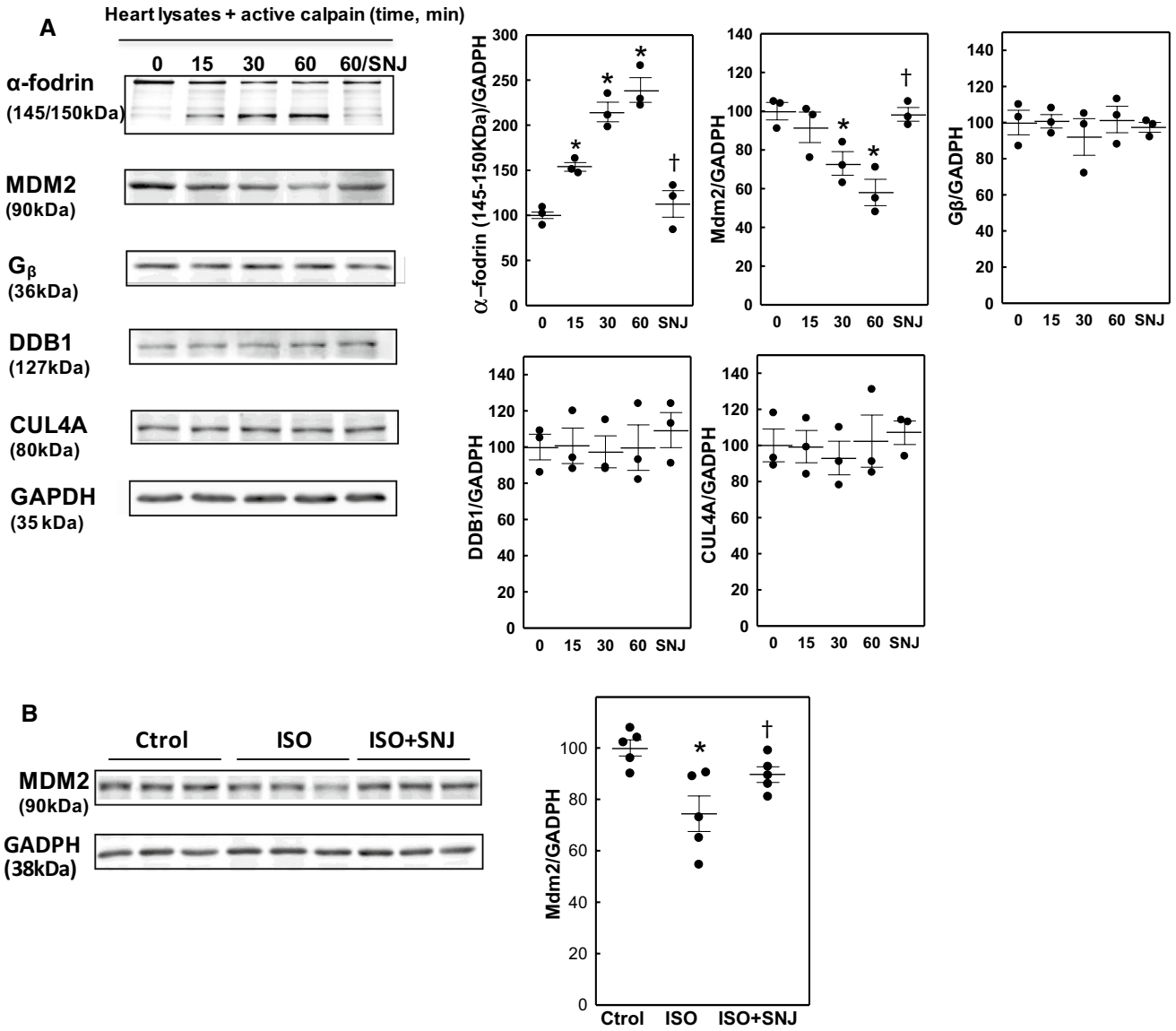

Fig. 5 Isoproterenol-induced calpain activation reduces myocardial MDM2 protein levels. **a** Myocardial rat extracts were incubated with recombinant calpain 1 with and without SNJ1945 for 0, 15, 30 and 60 min and analyzed for potential calpain-mediated cleavage of MDM2, Gβ, DDB1, and CUL4A by western blot. Calpain-dependent α-fodrin cleavage was used as a control. **b** MDM2 content in myocardial extracts obtained from control (Ctrol), isoproterenol (ISO) and isoproterenol + SNJ-1943 (ISO + SNJ) treated rat groups ($n = 5$ hearts per group) was assessed by western blot. Results are presented as mean ± SEM. *$p < 0.05$ vs. control group. Representative western blots are shown in both panels

We found that calpain-dependent upregulation of GRK2 protein levels involves mechanisms affecting both its degradation and synthesis (Fig. 8). GRK2 has been shown to be rapidly degraded by the proteasome pathway. Compelling evidence demonstrates that MDM2 is the main E3 ligase implicated in the ubiquitination and degradation of GRK2 upon GPCR activation [32, 40]. Our data show that isoproterenol treatment promotes a calpain-dependent decrease in cardiac MDM2 levels. The suitability of MDM2 as a calpain substrate is supported by the presence in the protein sequence of the E3 ligase of two canonical PEST sequences [15], which are regulatory determinants for calpain-mediated degradation of many proteins, and by a previous report suggesting that calpains participate in the downregulation of MDM2 in the epidermis after UV irradiation [8]. In addition, our data suggest that calpain activity might modulate MDM2 protein stability by promoting the proteolytic clearance of unidentified MDM2 regulatory factors, resulting in increased polyubiquitination patterns of MDM2 and its degradation. On the other hand, it has been described that G_β binds to DDB1 and targets GRK2 for ubiquitination by the DDB1-CUL4A-ROC1

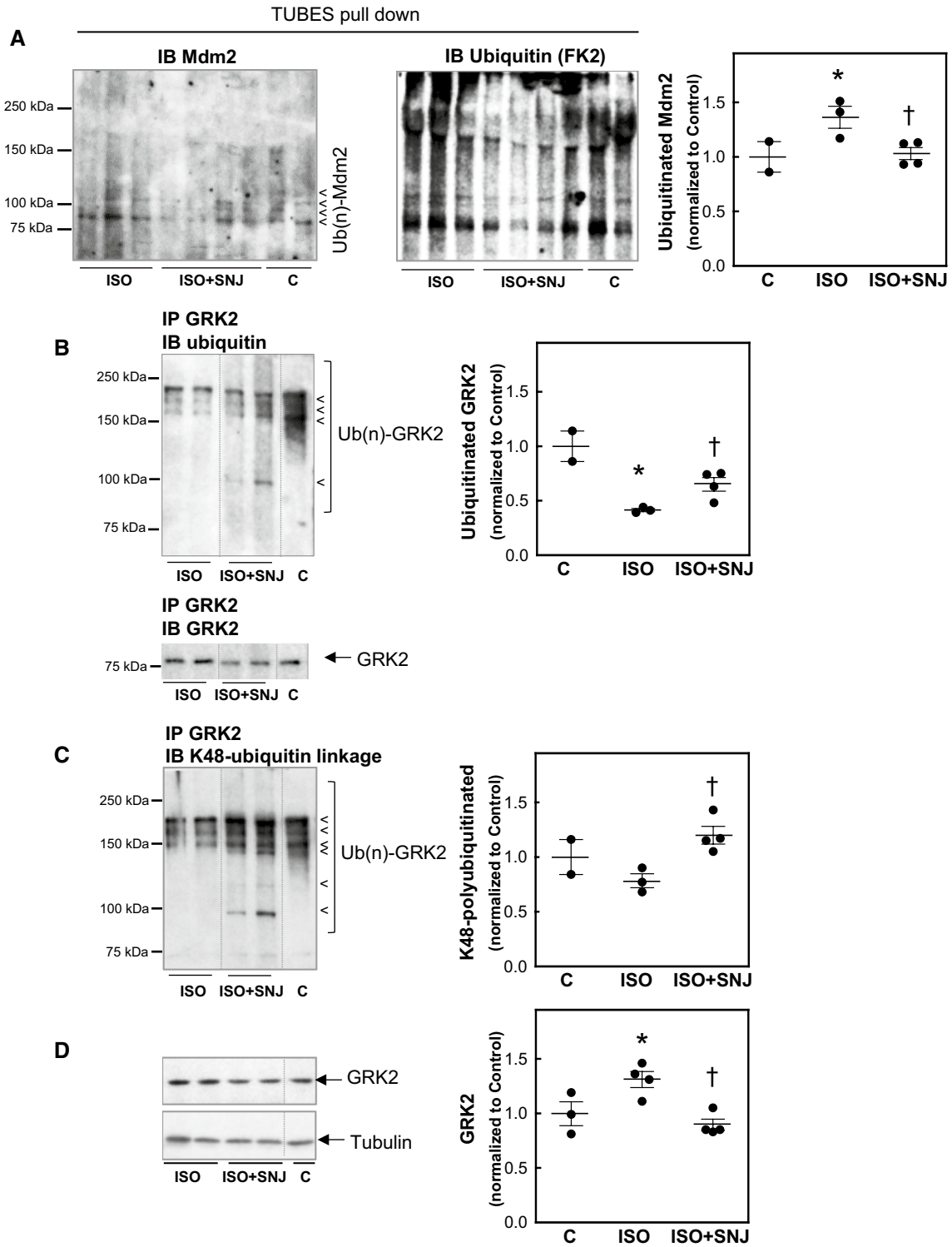

Fig. 6 Calpain inhibition decreases the extent of Mdm2 polyubiquitination and enhances polyubiquitination of GRK2 in isoproterenol-treated rats. **a** Polyubiquitination levels of Mdm2 in cardiac lysates from the indicated experimental conditions were analyzed using affinity-TUBEs pull-downs. Mdm2 bands were normalized to their corresponding ubiquitin smears and ratios were normalized to ubiquitinated Mdm2 level in control samples. **b** The same cardiac lysates used in panel C were analyzed for global GRK2 ubiquitination or, **c** specific (K)-48-linked polyubiquitination. The extent of both parameters inversely correlates with the total levels of GRK2 protein, **d** determined in these cardiac lysates by immunoblotting with a specific GRK2 antibody and normalized by tubulin as loading control. In panels **a**–**d** data indicate the mean ± SEM, $n = 2$–4 mice for each condition. Representative western blots are shown in all panels. Arrowheads indicate polyubiquitinated forms of ~ 100 Kda [Ub(3)-GRK2] and above (see text for details). *$p < 0.05$ vs. control group. †$p < 0.05$ vs. ISO group

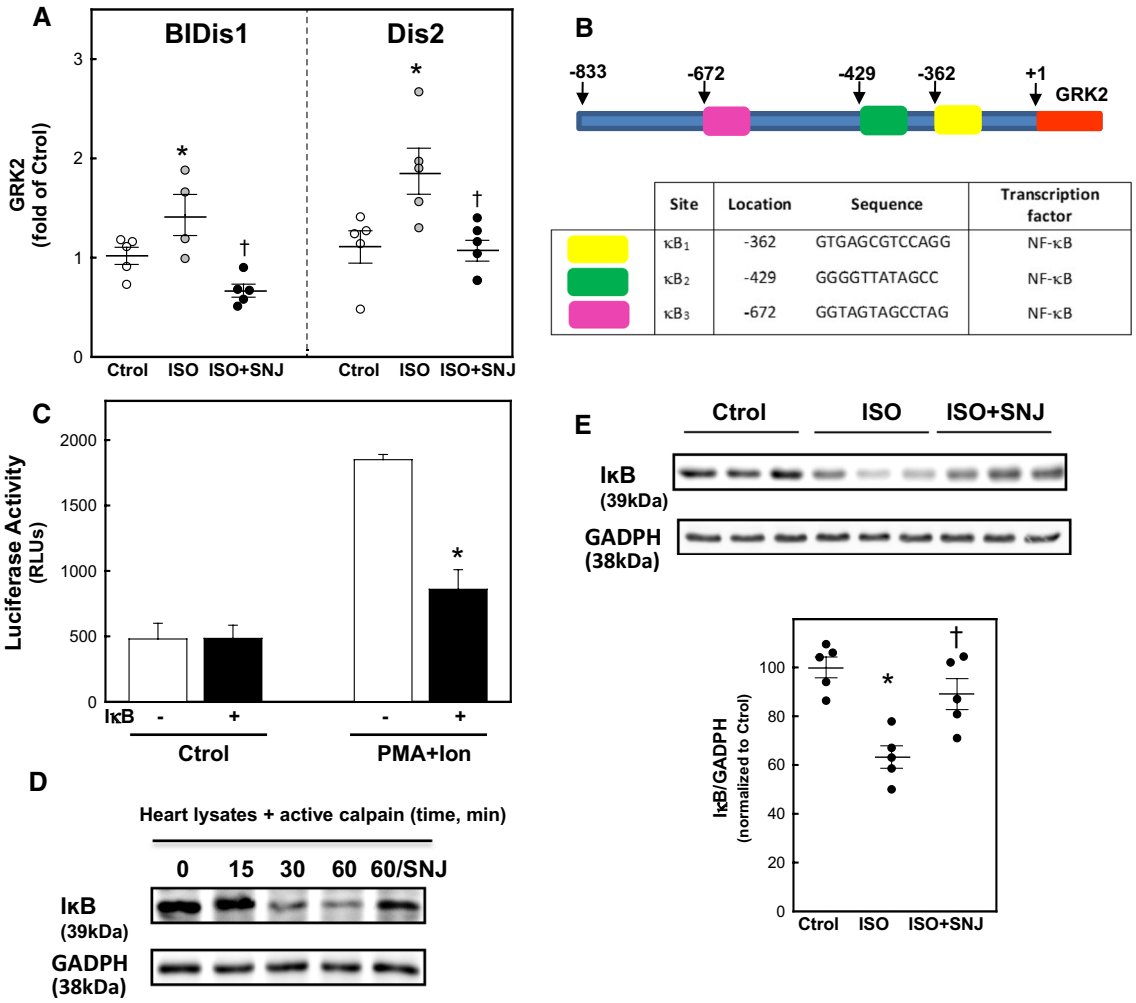

Fig. 7 Isoproterenol-induced calpain activation increases GRK2 gene expression by cleaving IκB. **a** Quantification of mRNA levels of GRK2 using two different primers ($n=5$ per group) in rat hearts obtained from the indicated experimental groups. Data are mean ± SEM. $^{*}p<0.05$ vs. control group. $^{\dagger}p<0.05$ vs. ISO group. **b** Scheme of the proximal 5´-flanking region of the human GRK2 gene promoter. Putative cis-acting consensus sequences for NFκB transcription factors are detailed and their relative position denoted by boxes. **c** Involvement of the NFκB pathway in the induction of GRK2 promoter activity. Human peripheral blood lymphocytes were co-transfected with the pGRK2-0.9 luciferase promoter construct plus an empty vector or an expression vector for the IκBα protein. Transfected cells were treated with PMA plus the Ca2 + Ionophore A23187. Luciferase activity is expressed as RLUs ± SEM. Similar results were obtained with cells from three different donors. $^{*}p<0.01$ vs. corresponding control group. **d** Myocardial rat extracts were incubated with recombinant calpain 1 with and without SNJ1945 for 0, 15, 30, and 60 min and analyzed for calpain-mediated IκB cleavage as in Fig. 5a. **e** IκB content in myocardial extracts obtained from control (Ctrol), isoproterenol (ISO) and isoproterenol + SNJ-1943 (ISO+SNJ)-treated rat groups ($n=5$ hearts per group) was assessed by western blot. Data are mean ± SEM. $^{*}p<0.05$ vs. control group. $^{\dagger}p<0.05$ vs. ISO group. Representative western blots are shown in panels **d** and **e**

E3 ligase complex [52]. The same group proposed that isoproterenol stabilizes GRK2 via a PKA-dependent reduction in the association between G_{β} and the DDB1-CUL4A complex. However, although our results do not rule out the participation of this cascade by indirect ways, the levels of these potential regulators were not affected by calpain overactivation, pointing instead to MDM2 as the downstream factor underlying isoproterenol-induced upregulation of GRK2 protein. Consistent with this notion, the extent of bulk and of specific (K)-48-linked GRK2 polyubiquitination inversely correlated with the total levels of GRK2 protein in isoproterenol and isoproterenol plus calpain inhibitor-treated rat groups, in coherence with the patterns of MDM2 expression in those groups. On the other hand, it is intriguing that myocardial GRK2 is not proteolytically processed by active calpains in conditions of chronic isoproterenol treatment, despite its reported partial degradation by calpain-2 in other cellular contexts [24]. Cardiac hypertrophy signaling pathways may turn GRK2 resistant to degradation by calpains by either

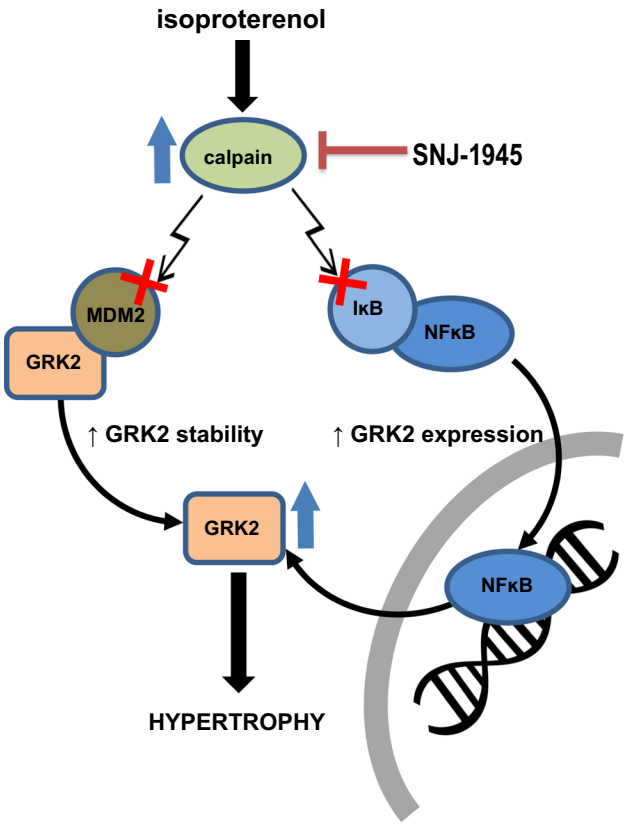

Fig. 8 Calpain activation modulates myocardial GRK2 protein levels by mechanisms affecting both its degradation and synthesis. Chronic isoproterenol treatment results in calpain activation which contributes to myocardial hypertrophy by upregulating GRK2 protein content. In the one hand, calpain reduces the levels of MDM2 thus increasing the stability of GRK2. In the other hand, calpain cleavages IκB therefore activating NFκB which in turn increases GRK2 gene transcription. Oral administration of SNJ-1945 is an effective strategy to prevent these effects

triggering "protective" GRK2 post-transcriptional modifications or by keeping away GRK2 from active calpains.

In sum, we propose that the calpain-dependent degradation of MDM2 would contribute to the enhanced GRK2 levels in isoproterenol-treated hearts as a result of increased GRK2 stabilization. Endogenous GRK2 levels increase in mouse embryonic fibroblasts lacking MDM2 [40], whereas both global or cardiomyocyte-specific deletion of MDM2 in mice led to a significant increase in cardiac GRK2 levels [17]. Importantly, conditional cardiomyocyte MDM2 deletion results in severely impaired cardiac function and decreased beta-adrenergic receptor responsiveness, which could be rescued upon delivery of a GRK2 inhibitor [17]. suggesting a relevant role for the MDM2/GRK2 regulatory axis in heart function. In this line, other studies have associated the downregulation of MDM2 with the development of cardiac hypertrophy induced by phenylephrine or endothelin-1 [46].

Our results also demonstrate that isoproterenol upregulates the synthesis of GRK2 by a calpain-dependent transcriptional mechanism. Although transcriptional upregulation of GRK2 and GRK5 isoforms has been reported in several cardiovascular pathologies, including hypertrophy, the underlying mechanisms have not been explored in detail. Our data put forward the NFκB cascade as a relevant calpain-dependent axis for enhancing GRK2 mRNA levels upon chronic isoproterenol treatment. NFκB activation in human lymphocytes, a bona fide model for changes in cardiomyocyte GRK2 expression [39], promotes a marked increase in the transcriptional activity of the proximal GRK2 promoter, which displays several canonical binding sites for this factor, whereas the presence of extra IκB significantly attenuates it.

On the other hand, the NFκB regulator IκB is a well-known calpain substrate and its calpain-dependent degradation has been associated to the activation of NFκB and the development of post-infarction remodeling [26, 34]. Our data demonstrate that calpain activation induced by chronic isoproterenol treatment also reduces myocardial IκB levels. Altogether, these results suggest a novel mechanism involved in the regulation of myocardial GRK2 expression in which calpain activation results in IκB protein degradation, in turn leading to NFκB activation and upregulation of GRK2 expression (Fig. 8).

The NFκB signaling pathway has been shown to play a critical role in the regulation of cardiac GRK5 expression at the level of gene transcription, promoting stress-induced GRK5 upregulation [16], and treatment of aortic vascular smooth cells with PMA reportedly increased the mRNA content of GRK2 [38], suggesting that the NFκB pathway might be a common trigger for maladaptive enhanced expression of cardiovascular GRKs, although regarding GRK2 the participation of other transcription factors downstream the isoproterenol/calpain axis cannot be ruled out.

A large amount of evidence suggests that NFκB plays a key role in cardiac hypertrophy induced by chronic stress [10, 19], and this nuclear factor has been related to isoproterenol-induced cardiac hypertrophy and remodeling [5]. The expression of a mutant IκB that acts as a super-repressor of NFκB in transgenic mice-attenuated hypertrophy induced by isoproterenol or angiotensin II infusion [5]. Since it has been proposed that GRK2 can activate NFκB and induce maladaptative hypertrophy [43], it is tempting to suggest that myocardial GRK2 and NFκB activity may co-regulate each other to trigger hypertrophic gene transcriptional activation. Upregulation of cardiac calpains [34] and GRK2 [12] and calpain-dependent activation of NFκB [34] have also been described during post-infarction remodeling. Hence, our results strongly suggest that the calpain/GRK2 axis may also be relevant in hypertrophy of ischemic cause.

The fact that calpains are overactivated in response to numerous stimuli associated to the development of hypertrophy suggests that calpain activation is a general response of cardiomyocytes to chronic stress. Despite that, pharmacological calpain inhibition has barely been considered as a therapeutic strategy for the treatment of ventricular remodeling [49]. Our study shows that sustained calpain inhibition is a promising novel treatment for preventing the development of hypertrophy. Although either oral or intraperitoneal administration of SNJ-1945 for 14 days produced no obvious toxicity or abnormalities in rats [30, 34], longer term and more detailed analysis of side effects are needed.

In conclusion, our study shows that chronic isoproterenol administration induces calpain overexpression and activation, which contributes to isoproterenol-induced myocardial hypertrophy by mechanisms involving GRK2 upregulation. Furthermore, our results unveil novel mechanisms affecting the regulation of myocardial GRK2 levels (Fig. 8). The present data demonstrate that the role of calpains in ventricular hypertrophy goes beyond post-infarction remodeling and suggest that sustained pharmacological inhibition of calpains may be an effective strategy to limit myocardial hypertrophy induced by chronic stress of both ischemic and non-ischemic origin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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